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13. ABSTRACT ***(Maximum 200 Words)***

Tumor invasion is a major obstacle to effective clinical management of breast cancer. To identify new targets for anti-invasive therapies, we have focused on the mechanisms by which the cell adhesion molecule E-cadherin suppresses tumor invasion. We previously found that E-cadherin does not suppress invasion via its adhesive activity. We hypothesized instead that E-cadherin-mediated contact generates intracellular signal(s) that regulate cell movement. In the present work, we have identified pathways by which E-cadherin affects cell behavior. In one pathway, cell-cell adhesion mediated by E-cadherin allows binding between the receptor tyrosine kinase EphA2 and its ligands. This interaction results in tyrosine phosphorylation of EphA2, which inhibits proliferation and decreases focal adhesions. The second pathway is defined by preliminary evidence suggesting that E-cadherin induces tyrosine phosphorylation of unidentified cell components by an EphA2-independent mechanism. It will now be important to identify components of both pathways and determine whether either pathway is responsible for suppression of cell movement by E-cadherin.

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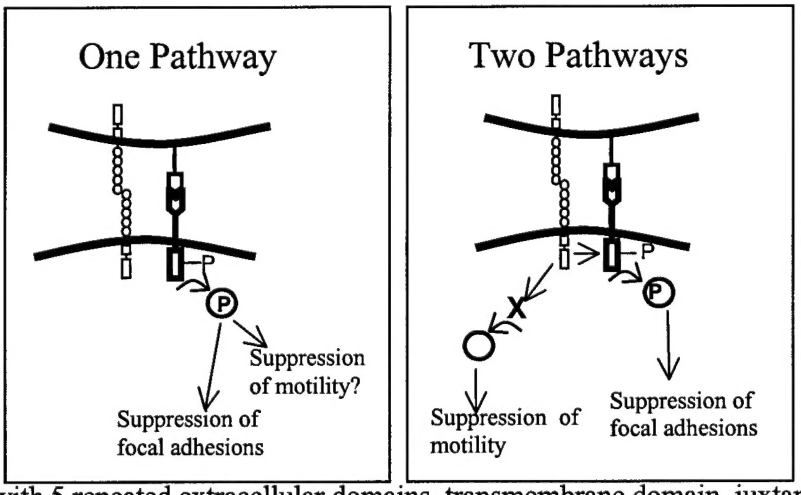
INTRODUCTION

Failures in treatment of breast tumors generally result from complications caused by tumor invasion and metastasis. This project aims to analyze mechanisms that regulate movement and invasion of mammary epithelial cells, with the ultimate goal of developing new anti-invasive therapies. One causal event in the acquisition of invasive capacity during breast tumor progression is loss of the cell-cell adhesion molecule, E-cadherin. We found previously that the ability of E-cadherin to suppress cell movement and invasion is not related to its adhesive activity. Instead, we hypothesize that cell-cell contact mediated by E-cadherin generates signals that suppress cell movement. We are testing this hypothesis and identifying components of the E-cadherin signaling system. We use molecular biological techniques to express different forms of E-cadherin in breast cancer cell lines, we analyze signaling events that are triggered by E-cadherin using biochemical approaches, and we test the effect of E-cadherin on cell behavior through time-lapse videorecording.

BODY

As described below, we have made substantial progress toward our overall goals. Some initial findings required us to make significant changes, however, in our original plans.

A t the outset of the period of funded research, our collaborator, Dr. Michael Kinch, identified one of the components of the E-cadherin-dependent tyrosine phosphorylation cascade as the receptor tyrosine kinase EphA2 (Kinch *et al.*, 1998). This was an important discovery, because it immediately suggested specific models for E-cadherin signaling. Ligand(s) for EphA2, termed Ephrins, are located at the cell surface. Thus, contact mediated by E-cadherin could bring together EphA2 and a ligand, resulting in EphA2 activation and phosphorylation of substrate proteins that regulate cell movement. This could be the sole tyrosine phosphorylation pathway triggered by E-cadherin (One Pathway model below). It is also possible that E-cadherin might stimulate one or more avenues of tyrosine phosphorylation in addition to the EphA2 pathway (Two Pathways model below).



E-cadherin with 5 repeated extracellular domains, transmembrane domain, juxtamembrane domain and catenin-binding domain

EphA2 with extracellular ligand-binding domain, transmembrane domain and intracellular kinase domain

Ephrin with GPI linkage to membrane and EphA2-binding domain

We therefore placed emphasis upon establishing the relationship between E-cadherin expression and EphA2 activation and ultimately upon testing whether the Single Pathway or Two Pathways models are more accurate. Experiments that address these issues are described under Technical Objective 2, task 1 (months 4-10) and Technical Objective 3, task 11 (originally planned for months 18-36). In addition, as described later, identification of EphA2 had consequences that altered plans described in Technical Objective 1, tasks 1 and 2. For the convenience of the readers, we have first summarized our progress in narrative form with references to the Technical Objectives, followed by a listing of the Technical Objectives in order, with capsule summaries of work accomplished toward each Objective.

Narrative Description of Work Accomplished

Initial studies to determine the relationship between E-cadherin expression and EphA2 activation are described in an appended manuscript that is currently in press (Zantek *et al.*, 1999). Briefly, this work demonstrates that E-cadherin is required for tyrosine phosphorylation of EphA2. Relatively normal mammary epithelial cells express E-cadherin and EphA2, which is tyrosine phosphorylated. Inhibition of E-cadherin activity in these cells abolishes tyrosine phosphorylation of EphA2. Metastatic breast cancer lines, such as MDA-MB-231 express EphA2 but do not express E-cadherin. In these cells, EphA2 is not tyrosine phosphorylated, but phosphorylation is restored by expression of E-cadherin. The presence or absence of tyrosine phosphorylation had significant consequences, as we found that tyrosine phosphorylation of EphA2 inhibits focal adhesions and cell proliferation. Inhibition of focal adhesions by EphA2 could be a significant part of E-cadherin's effect on cell movement.

The involvement of EphA2 in E-cadherin signaling raised a significant question that related to our earlier work. We previously found that suppression of cell movement by E-cadherin did not require the adhesive function of E-cadherin (Chen *et al.*, 1997). In contrast, tyrosine phosphorylation of EphA2 and resultant inhibition of focal adhesions relies on the adhesive activity of E-cadherin to bring EphA2 and its ligand into close proximity (Zantek et al., 1999). Several explanations are possible for this apparent discrepancy. One possibility is that EphA2 activation is not the sole E-cadherin signaling pathway (e.g., the Two Pathways model shown above). To determine whether E-cadherin affects movement via one or two pathways, it is necessary to test the effect of E-cadherin contact *in the absence of EphA2 signaling.*

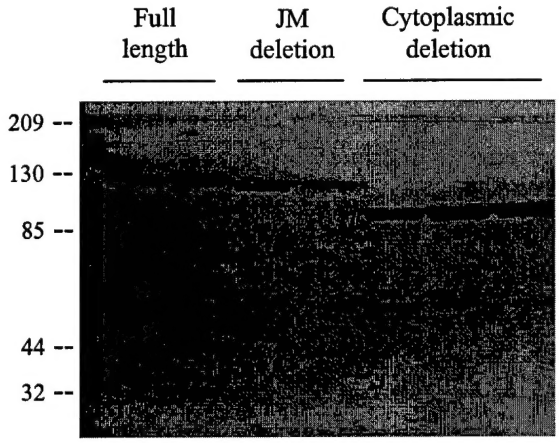
We have begun two approaches, not proposed in the original application, toward this end. First, we produced a mutant form of EphA2, in which a K646A transition renders the kinase inactive. We anticipate that this mutant EphA2, like kinase-inactive mutants of other receptor tyrosine kinases, should function as a dominant-negative inhibitor that would squelch activity of endogenous EphA2 when introduced into cells. This will allow us to test whether E-cadherin still generates signals when EphA2 activity has been suppressed.

As a second approach, we developed a "clustering assay" that triggers E-cadherin signaling in the absence of cell-cell contact, thus preventing interactions between EphA2 and its ligands. In the "clustering assay," antibodies are added to live, isolated cells to induce clustering of E-cadherin, as occurs during cell-cell adhesion. In initial experiments using this assay, we have found that clustering E-cadherin induces clusters of phosphotyrosine that are co-localized with E-cadherin clusters. In the experiment shown on the following page, full-length E-cadherin in MDA-MB-231 cells was clustered by addition of antibodies to E-cadherin followed by secondary antibodies. The cells were then fixed, permeabilized and stained for phosphotyrosine. Using confocal microscopy, induced clusters of E-cadherin are seen at the cell surface and many of these clusters are associated with colocalized phosphotyrosine (see photomicrograph). Although not shown, we verified that


green E-cadherin
red phosphotyrosine
yellow colocalization

EphA2 remained dispersely distributed during this experiment and, thus, was not contributing to the observed clusters of phosphotyrosine. These experiments suggest the existence of an EphA2-independent signaling pathway, thus supporting the Two Pathways model. Preliminary experiments suggest that the cytoplasmic domain of E-cadherin is not required for co-clustering of phosphotyrosine, but all of these findings must be repeated and confirmed.

Another important Objective from the original application was to determine whether the juxtamembrane domain was required for tyrosine phosphorylation triggered by E-cadherin-mediated cell-cell contact. To accomplish this aim, we proposed to generate a series of cell lines that express full-length E-cadherin or mutant forms lacking various portions of the cytoplasmic domain and then to analyze tyrosine phosphorylation in these cells (Technical Objective 1, task 2 and Technical Objective 2, tasks 1 and 2). We have nearly completed production of these cell lines. The immunoblot reproduced below documents expression of the different mutant forms of E-cadherin in the selected lines.

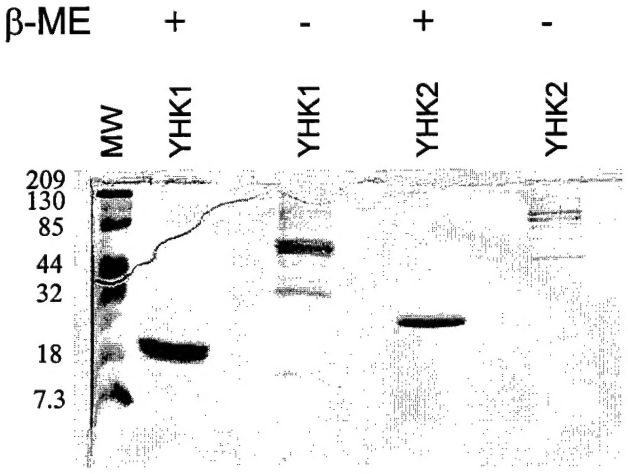


Initially, we will assess tyrosine phosphorylation in these cells via the calcium-switch paradigm described in Zantek et al., 1999. Because this assay depends on cell-cell adhesion, however, it is not possible to determine whether a particular mutation alters signaling directly, or as a consequence of effects on adhesion. The clustering assay described above mimics adhesion without cell-cell contact and can therefore be used to assay the effect of clustering all forms of E-cadherin.

Another major goal of our proposed studies was to identify components that react with the juxtamembrane region of E-cadherin (Technical Objective 3, tasks 3 and 7). In initial co-immunoprecipitation studies (Technical Objective 3, task 3), we tested three different antibodies against E-cadherin and determined that two of them, the DECMA rat monoclonal antibody (which is commercially available, but expensive) and a rabbit polyclonal antibody that we generated (#795), reacted with both full-length and JM-domain-deleted forms. We have found that turnover of E-cadherin in MDA-MB-231 cells is slow, making it difficult to incorporate sufficient amounts of radioactivity for visualization of components that bind to E-cadherin in sub-stoichiometric amounts. We have, therefore, turned to large-scale immunoaffinity purification of unlabeled E-cadherin and associated components and identification of the associated components by mass spectrometry. These experiments are in progress.

In a second approach to identifying components that react with the juxtamembrane region of E-cadherin (Technical Objective 3, task 7), we are using GST fusion proteins contains portions of the juxtamembrane region. Initial experiments using monomeric fusion proteins did not reveal any binding components. We

believe, however, that the JM domain may need to be clustered before association of signaling components, as is the case for integrins. We therefore obtained a vector from Dr. Mark Ginsberg (Pfaff *et al.*, 1998), designed to produce an integrin β cytoplasmic domain fusion protein that promoted the formation of integrin subunit dimers in vitro. This fusion contained a 6-his tag, 4 heptad repeats and a cysteine to allow disulfide bonding fused to the integrin β cytoplasmic domain. Using this construct, Pfaff *et al.*, were able to show in vitro interaction between integrin and talin. Since cadherin is thought to exist as a dimer or oligomer at the cell surface (Pertz *et al.*, 1999; Takeda *et al.*, 1999) we decided to engineer a JM fusion based on Ginsberg's construct. We made two fusions of the his tag, heptad repeats and cysteine with the E-cad JM region. One, YHK1, contains the first 53 amino acids of the E-cad cytoplasmic domain and the other, YHK2, contains the first 98 amino acids. Both fusion proteins contain a second cysteine at the carboxyl terminus, introduced by the cloning procedure. We have shown that these fusion proteins are expressed and can be purified from the soluble fraction of bacterial lysates on a nickel column. As illustrated below, the purified proteins are monomers when reduced by β-mercaptoethanol (β-ME) and oligomers in a non-reducing environment (see below). These proteins will be coupled to beads and used as affinity supports for purifying components that interact with the JM domain.



Finally, the identification of EphA2 as a key participant in E-cadherin signaling, caused us to delay carrying out experiments described in Technical Objective 1. We felt it was important first to screen the breast cancer cell lines for expression of EphA2, as well as cadherins. We found that the MDA-MB-435 strain we were using expressed N-cadherin, but not EphA2, whereas the MDA-MB-435 isolate used by the Kinch laboratory expressed EphA2, but not N-cadherin! We have, therefore transfected the Kinch MDA-MB-435 line with an N-cadherin expression vector and isolated stably transfected lines. We also have transfected MDA-MB-231 (which expresses EphA2 that is not phosphorylated) with an expression vector for N-cadherin and obtained permanently transfected lines. These lines will be characterized and then the assays described in Technical Objective 1 will be performed.

Summary of Work Accomplished on Revised Technical Objectives

Technical Objective 1: To verify that E- and N-cadherin differ in their ability to suppress invasion of mammary carcinoma cells and to use this difference to define regions of E–cadherin that are essential for suppressing invasion.

Task 1. Months 1-3. We will assay the invasiveness of MDA-MB-435S cells and verify their N-cadherin expression.

Task 2. Months 3-9. MDA-MB-435S cells and MDA-MB-231 cells will be transfected with control, E-cadherin, N-cadherin (in the case of MDA-MB-231 cells), and chimeric E/N cadherin vectors and with E-cadherin mutant vectors (for studies of Technical Objective 2). Permanent lines will be selected, re-cloned, and characterized for cadherin expression and adhesion.

Task 3. Months 9-15. The invasion and motility of the transfected lines will be evaluated. Each assay will be performed 3-5 times.

We examined the cell lines for expression of EphA2 and cadherins and have produced permanently-transfected lines of MDA-MB-231 that express full-length E- or N-cadherin and lines that express several cytoplasmic domain deletion forms of E-cadherin. Characterization of these lines is in progress.

Technical Objective 2 To determine whether an intact juxtamembrane domain is required for E-cadherin-induced tyrosine phosphorylation.

Task 1. Months 4-10. Work out assays and then complete final analyses of tyrosine phosphorylation in untransfected MDA-MB-435S cells *and MDA-MB-231 cells.*

To examine E-cadherin dependent tyrosine phosphorylation that is independent of EphA2, we developed the "clustering" assay.

Task 2. Months 12-24. Assay tyrosine phosphorylation in MDA-MB-435S cells *and MDA-MB-231 cells* transfected with full-length or mutant E-cadherins.

We have recently begun these experiments.

Technical Objective 3 To identify factors that interact with the juxtamembrane domain of E–cadherin.

Task 1 and Task 2. *These experiments were duplicative of the NIH award and were deleted.*

Task 3. Months 4-10. We will analyze, by co-immunoprecipitation studies, components that may be associated with E-cadherin, but not the JM-deleted form, in MDA-MB-435S cells and MDA-MB-231 cells.

These experiments are in progress.

Tasks 4 (Months 10-24)**, 5** (Months 6-15)**, and 6** (Months 16-36)**.**

No work yet initiated.

Task 7. Months 9-18. We will use GST-fusion proteins for affinity-purification of components associated with the JM domain.

We have produced JM domain fusion proteins that multimerize and will soon use these as supports in affinity chromatography.

Task 10. Months 18-24. We will produce antibodies against the affinity-purified components and begin testing for physiological interactions.

Task 11. Months 18-36. We will attempt to identify proteins that are tyrosine phosphorylated in response to E-cadherin binding and will begin to test whether they interact with E-cadherin.

No work yet initiated.

KEY RESEARCH ACCOMPLISHMENTS

- Identified the receptor tyrosine kinase EphA2 as one target of the E-cadherin signaling system
- Demonstrated that E-cadherin is required for EphA2 activation in mammary epithelial cells
- Demonstrated that EphA2 activation suppresses focal adhesions and represses proliferation of mammary epithelial cells
- Determined that E-cadherin mediated contact also induces tyrosine phosphorylation of proteins via an EphA2-independent mechanism

REPORTABLE OUTCOMES

Publications:

Zantek, N.D., Azimi, M., Hein, P., Fedor-Chaiken, M., Brackenbury, R., and M.S. Kinch. 1999. E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. *Cell Growth and Differentiation*, in press.

Cell Lines

We have produced variants of MDA-MB-231 transfected with expression vectors encoding full-length E- and N-cadherin and cytoplasmic domain deletion mutants of E-cadherin.

We have produced variants of MDA-MB-435 transfected with expression vectors encoding full-length E- and N-cadherin.

CONCLUSIONS

Cell-cell contact mediated by E-cadherin suppresses movement of mammary epithelial cells. We have identified intracellular pathways by which E-cadherin affects cell behavior. In one pathway, cell-cell adhesion mediated by E-cadherin allows binding between the receptor tyrosine kinase EphA2 and its ligands. This

interaction results in tyrosine phosphorylation of EphA2, which inhibits proliferation and focal adhesions. The second pathway is defined by preliminary evidence that suggests E-cadherin induces tyrosine phosphorylation of unidentified cell components by an EphA2-independent mechanism. It will now be important to determine whether either of these pathways is responsible for suppression of cell movement by E-cadherin. Characterization of these signaling pathways may identify new targets for anti-invasive therapies.

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E-Cadherin Regulates the Function of the EphA2 Receptor Tyrosine Kinase[1]

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Abstract

EphA2 is a member of the Eph family of receptor tyrosine kinases, which are increasingly understood to play critical roles in disease and development. We report here the regulation of EphA2 by E-cadherin. In nonneoplastic epithelia, EphA2 was tyrosine-phosphorylated and localized to sites of cell-cell contact. These properties required the proper expression and functioning of E-cadherin. In breast cancer cells that lack E-cadherin, the phosphotyrosine content of EphA2 was decreased, and EphA2 was redistributed into membrane ruffles. Expression of E-cadherin in metastatic cells restored a more normal pattern of EphA2 phosphorylation and localization. Activation of EphA2, either by E-cadherin expression or antibody-mediated aggregation, decreased cell-extracellular matrix adhesion and cell growth. Altogether, this demonstrates that EphA2 function is dependent on E-cadherin and suggests that loss of E-cadherin function may alter neoplastic cell growth and adhesion via effects on EphA2.

Introduction

Protein tyrosine phosphorylation generates the powerful signals necessary for the growth, migration, and invasion of normal and malignant cells (1). A number of tyrosine kinases have been linked with cancer progression (2), and increased tyrosine kinase activity is an accurate marker of cancer progression (3, 4). EphA2 (epithelial cell kinase) is a M_r 130,000 member of the Eph family of receptor tyrosine kinases (5), which interact with cell-bound ligands known as ephrins (1, 6, 7). Whereas EphA2 and most other Eph kinases are expressed and well studied in the developing embryo (8), in the adult, EphA2 is expressed predominantly in epithelial tissues (5). The function of EphA2 is not known, but it has been suggested to regulate proliferation, differentiation, and barrier function of colonic epithelium (9); stimulate angiogenesis (10); and regulate neuron survival (11). Little is known of EphA2's role in cancer, although recent studies demonstrate EphA2 expression in human melanomas (12), colon cancers (9), and some oncogene-induced murine mammary tumors (13).

There is much interest in how tyrosine kinases like EphA2 regulate cell growth and differentiation. One often unappreciated mechanistic hint is the observation that substrates of tyrosine kinases are found almost exclusively within sites of cellular adhesion (14). In epithelial cells, for example, tyrosine-phosphorylated proteins are predominantly located in E-cadherin-associated adherens junctions (14, 15). E-cadherin mediates calcium-dependent cell-cell adhesions through homophillic interactions with E-cadherin on apposing cells (16, 17). In cancer cells, E-cadherin function is frequently destabilized, either by loss of E-cadherin expression (18) or by disruption of linkages between E-cadherin and the actin cytoskeleton (19–23). Restoration of E-cadherin function, either by E-cadherin transfection (24, 25) or treatment with pharmacological reagents (21), is sufficient to block cancer cell growth and induce epithelial differentiation. However, the mechanisms by which E-cadherin imparts these tumor suppressor functions are largely unknown. Whereas E-cadherin-mediated stabilization of cell-cell contacts undoubtedly is involved, there is recent evidence that E-cadherin also generates intracellular signals that could contribute to tumor suppression (15, 26, 27).

Previous studies by our laboratory have linked E-cadherin with signaling by tyrosine phosphorylation. E-cadherin aggregation into assembling adherens junctions initiates a signaling cascade involving tyrosine phosphorylation that may contribute to E-cadherin's tumor suppressor function (28). In addition, we have demonstrated that transformed epithelial cells have elevated levels of tyrosine phosphorylation that destabilize E-cadherin function (21). To identify tyrosine kinases and their substrates in breast cancer, we recently generated monoclonal antibodies that are specific for tyrosine-phosphorylated proteins in Ras-transformed breast epithelial cells (15). Using these antibodies, we identified the EphA2 tyrosine kinase as a protein that is tyrosine-phosphorylated upon E-cadherin-mediated adhesion. We also show that E-cadherin regulates the functioning of EphA2.

Results

Regulation of EphA2 Expression in Breast Cancer Cells. We measured EphA2 expression levels in breast epithelial cell lines derived from nonneoplastic epithelia (*e.g.*, MCF-

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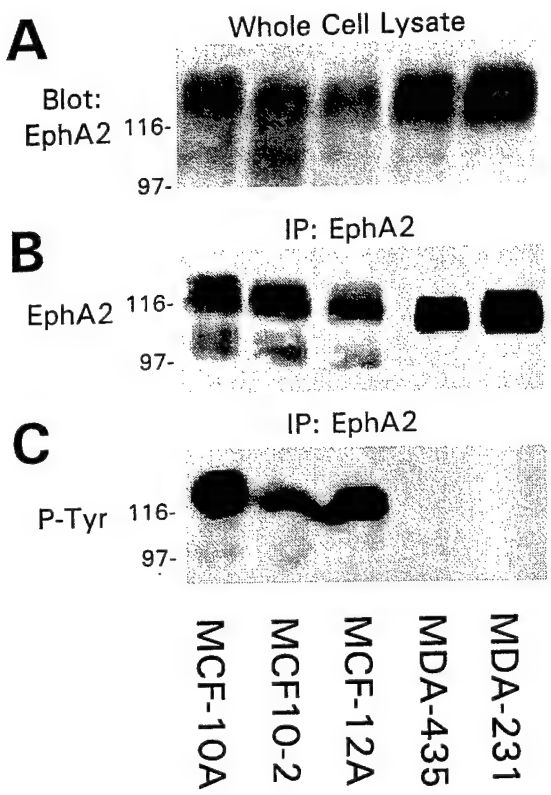

Fig. 1. Decreased EphA2 phosphorylation in metastases. EphA2 from whole cell lysates (*A*) or immunoprecipitated from monolayers of nonneoplastic (MCF-10A, MCF10-2, and MCF-12A) and metastatic (MDA-MB-231 and MDA-MB-435) breast cancer cell lines (*B*) was resolved by SDS-PAGE and Western blot analysis performed with EphA2 antibodies. *C*, the blot from *B* was stripped and reprobed with phosphotyrosine-specific (PY20) antibodies. Note the absence of tyrosine-phosphorylated EphA2 in metastatic breast cancer cells.

10A, MCF-12A, and MCF10-2; Refs. 29 and 30) and metastatic breast cancer (*e.g.*, MDA-MB-231 and MDA-MB-435; Refs. 31 and 32). EphA2 was found to be expressed in nontransformed mammary epithelial and metastatic breast cancer cell lines tested (Fig. 1*A* and data not shown), with 2–5-fold more EphA2 in neoplastic cells, as determined by Western blot analysis using multiple EphA2 antibodies and by Northern blot analysis (data not shown).

Despite its overexpression, EphA2 in metastatic cells displayed a much-reduced phosphotyrosine content. For these studies, EphA2 was immunoprecipitated from confluent monolayers of either nonneoplastic or metastatic cells and Western blot analysis performed with phosphotyrosine specific antibodies. This revealed prominent phosphorylation of EphA2 in nonneoplastic cells, whereas the EphA2 from metastatic cells was not tyrosine-phosphorylated (Fig. 1C). The decreased phosphotyrosine content was confirmed using different EphA2 antibodies (D7, B2D6, and rabbit polyclonal antibodies) for immunoprecipitation and with multiple phosphotyrosine antibodies (PY20, 4G10, and rabbit polyclonal antibodies) for Western blot analysis (data not shown). Decreased EphA2 phosphorylation was also observed in other metastatic breast cancer cell lines as well as invasive tumor

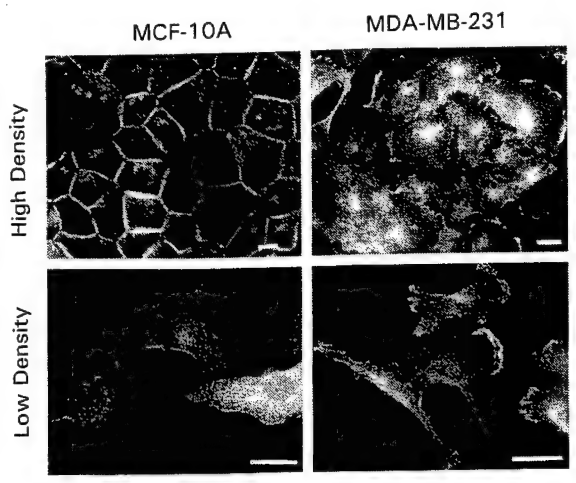

Fig. 2. Altered EphA2 localization in metastatic cancer cells. The subcellular distribution of EphA2 in nontransformed mammary epithelial cells (MCF-10A) and metastatic breast cancer cells (MDA-MB-231) was assessed by immunostaining with EphA2-specific antibodies. The cells were plated at either high (*top*) or low (*bottom*) cell density to emphasize the localization of EphA2 within cell-cell contacts or membrane ruffles of nontransformed or invasive cells, respectively. *Scale bars*, 10 μm.

cell lines derived from colon, pancreatic, ovarian, and lung cancers (data not shown).

Further comparison of EphA2 in nonneoplastic and metastatic cells revealed other changes in EphA2 distribution and function. Immunofluorescence staining with EphA2-specific antibodies revealed that EphA2 in nonneoplastic cells was mostly found within sites of cell-cell contact (Fig. 2), with little staining of membrane that was not in contact with neighboring cells. In contrast, EphA2 in metastatic cells was absent from sites of cell-cell contacts. Instead, the EphA2 in these cells was either diffusely distributed or enriched within membrane ruffles at the leading edge of migrating cells. The enrichment within membrane ruffles was confirmed by colocalization of EphA2 with f-actin (data not shown). This localization within membrane ruffles was not observed in nontransformed epithelia, even at low cell density. These differences in subcellular distribution were confirmed using three different EphA2-specific antibodies (D7, B2D6, and rabbit polyclonal antibodies). The correlation between EphA2 localization and phosphotyrosine content forms the basis for much of the remainder of this study.

EphA2 Enzymatic Activity in Metastatic Cells. Tyrosine phosphorylation of a kinase often regulates enzymatic activity. To test the effect of differences in EphA2 phosphorylation on kinase activity, we measured EphA2 autophosphorylation by using *in vitro* kinase assays with immunoprecipitated material (Fig. 3). Despite the low phosphotyrosine content of EphA2 in metastatic cells, this EphA2 demonstrated enzymatic activity that was comparable with or higher than the activity of EphA2 isolated from nonneoplastic cells. This activity was unaffected by the basal phosphotyrosine content of EphA2 because unlabeled phosphate was rapidly exchanged with labeled phosphate during the autophosphorylation assays as described previously (33, 34). KOH treatment of the membranes prior to autoradiography did not

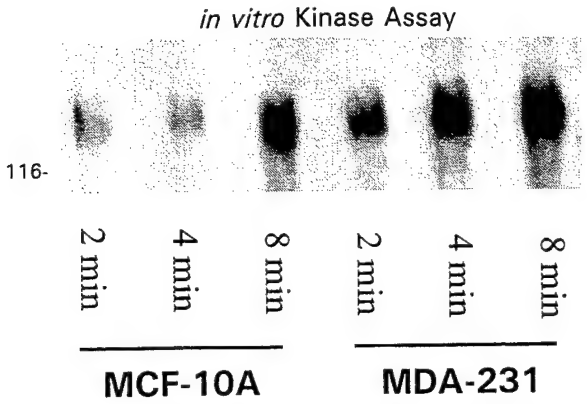

Fig. 3. EphA2 enzymatic activity. The enzymatic activity of EphA2 was measured using an *in vitro* autophosphorylation assays. At the times shown, the *in vitro* reaction was terminated and resolved by SDS-PAGE. The blot shown was treated with KOH to hydrolyze phosphoserine and phosphothreonine prior to autoradiography. After several half-lives, Western blot analysis was performed with EphA2 antibodies to confirm equal sample loading (data not shown).

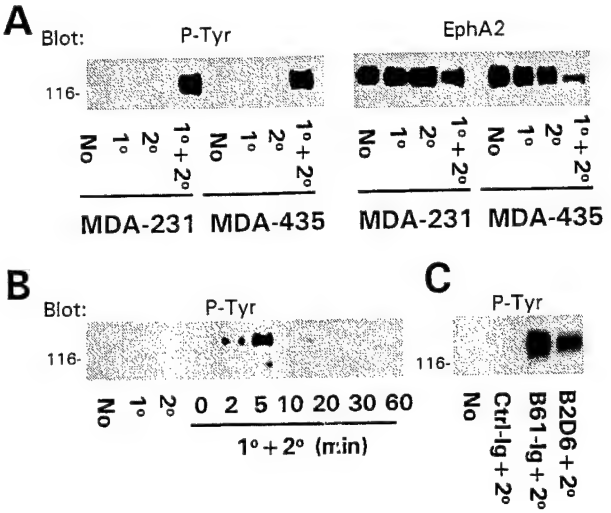

Fig. 4. Antibody-mediated aggregation induces EphA2 phosphorylation in metastatic cells. *A*, immunoprecipitated EphA2 was subjected to Western blot analysis with phosphotyrosine antibodies (PY20) following aggregation of cell surface EphA2 for 5 min at 37°C with specific primary and secondary antibodies (*1°+2°*). Note that simple engagement of anti-EphA2 (*1°*) or antimouse (*2°*) alone was insufficient to induce tyrosine phosphorylation above basal levels (*No*). The blot was then stripped and reprobed with EphA2 antibodies as a loading control. *B*, the time course of EphA2 phosphorylation was measured after cross-linking (*1°+2°*) EphA2 in MDA-MB-231 cells for 0–60 min by Western blot analysis of immunoprecipitated EphA2 with phosphotyrosine-specific antibodies (PY20). *C*, EphA2 was aggregated using a soluble ligand fusion protein (B61-IgG). A control fusion protein (Ctrl-IgG) served as a negative control, and B2D6-mediated aggregation served as a positive control for activation.

significantly reduce the level of phosphorylation, indicating that the observed enzymatic activity represented mostly phosphorylation on tyrosine residues. It is also notable that the phosphotyrosine content of EphA2 (Fig. 1*B*) was not predictive of its enzymatic activity (Fig. 3).

Receptor Aggregation Induces EphA2 Tyrosine Phosphorylation in Metastatic Cells. EphA2 in neoplastic cells retained the capacity to become activated. For example, EphA2 tyrosine phosphorylation was induced by aggregation of EphA2 with a soluble form of ephrin-A (B61-IgG, a chimera of the EphrinA1 extracellular domain fused to immunoglobulin heavy chain; also known as a "ligand-body"; Refs. 10 and 35; Fig. 4*C*). In contrast, a control chimera (Ctrl-IgG) did not alter EphA2 phosphorylation. Clustering EphA2 at the cell surface with specific antibodies (EK166B or B2D6) also induced levels of EphA2 activation that were comparable with that nonneoplastic cells (Fig. 4*A*). Receptor aggregation, not simply antibody binding, was necessary for EphA2 phosphorylation as incubation with anti-EphA2 (Fig. 4, *1°*) alone did not increase EphA2 phosphorylation relative to matched controls. This effect was specific for EphA2 as neither secondary (Fig. 4, *2°*) antibodies alone or clustering of isotype-matched control antibodies (which recognize an inaccessible cytoplasmic epitope on EphA2) did not induce tyrosine phosphorylation of EphA2 (data not shown). Analysis of the timing of EphA2 phosphorylation revealed EphA2 phosphorylation within 2 min after cross-linking, with optimal phosphorylation detected after 5 min (Fig. 4*B*).

E-Cadherin Regulates EphA2 in Nontransformed Epithelia. Tyrosine phosphorylation of EphA2 correlates with its localization within sites of cell-cell contact. Because Eph receptors become activated by ligands that are attached to the surface of neighboring cells (36), we reasoned that stable cell-cell adhesions might be necessary for EphA2 activation. Adhesions mediated by E-cadherin generate the most stable interactions between epithelial cells (16), and we noted that EphA2 was not phosphorylated and was absent from intercellular contacts in cells lacking E-cadherin. These include metastatic cancer cells as well as nontransformed fibroblasts (*e.g.*, NIH 3T3, REF-52, and C3H10T½) and myoepithelial cells (HBL-100; data not shown). We, therefore, tested whether E-cadherin might regulate EphA2 phosphorylation.

Because both EphA2 and E-cadherin are found at sites of cell-cell contact, we first examined whether the two proteins colocalize using two-color immunofluorescence microscopy. This revealed an overlapping distribution of EphA2 and E-cadherin along the lateral membranes of epithelial cells and, specifically, within sites of cell-cell contact (Fig. 5). Vertical sectioning by confocal microscopy confirmed colocalization of E-cadherin and EphA2 within sites of cell-cell contact (data not shown).

To test whether the colocalization of EphA2 and E-cadherin might indicate a functional link between the two proteins, we disrupted calcium-dependent E-cadherin-mediated adhesion by supplementing the cell culture medium with 4 mM EGTA, a calcium-chelating agent. EGTA treatment caused EphA2 dephosphorylation (Fig. 6*A*) and induced either a diffuse or membrane ruffle pattern of staining (Fig. 6*C*), which was reminiscent of EphA2 in metastatic cells. Subsequent restoration of normal levels of extracellular calcium restored normal levels of EphA2 phosphorylation and cell-cell localization within 5 min (Fig. 6, *A* and *C*).

Although results with EGTA-treated samples implicate cell-cell adhesion with the control of EphA2 phosphorylation

Appendix

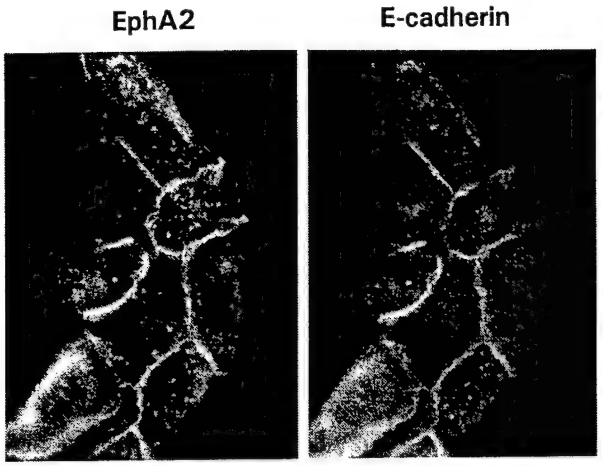

Fig. 5. Colocalization of EphA2 and E-cadherin. The subcellular distribution of EphA2 (*left*) and E-cadherin (*right*) was evaluated in MCF-10A cells using two-color immunofluorescence microscopy. Note the overlapping distribution of EphA2 and E-cadherin within sites of intercellular junctions.

and subcellular localization, we sought to determine whether E-cadherin contributed to this regulation. For this, we supplemented the cell culture medium with function-blocking E-cadherin antibodies and peptides (DECMA-1 antibodies or HAV peptides; Refs. 37 and 38). When inhibitors of E-cadherin function were added to the medium concomitant with the restoration of extracellular calcium, EphA2 did not become tyrosine-phosphorylated (Fig. 7*A*) and remained diffuse or present within membrane ruffles (Fig. 7*C*). In contrast, isotype-matched control antibodies and scrambled peptides did not prevent EphA2 phosphorylation or localization within intercellular junctions. Specific inhibition of E-cadherin with these inhibitors also blocked EphA2 phosphorylation and cell-cell localization upon treatment of confluent cell monolayers (data not shown), thus confirming that EphA2 phosphorylation and localization are sensitive to the functioning of E-cadherin.

EphA2 Is Responsive to E-Cadherin Expression in Metastatic Cells. To examine further the link between EphA2 and E-cadherin, we transfected MDA-MB-231 cells with E-cadherin (231-E-cad) and selected for levels of E-cadherin expression that were equivalent to MCF-10A cells. As controls, we transfected cells with empty vector (231-neo). EphA2 in 231-neo was not phosphorylated and was enriched within membrane ruffles (Fig. 8). In contrast, the EphA2 in 231-E-cad redistributed into sites of cell-cell contacts and had levels of phosphotyrosine that were comparable with that of MCF-10A cells (Fig. 9*A*). These changes in EphA2 phosphorylation and localization increased with cell density (data not shown), consistent with an idea that E-cadherin function regulates EphA2 phosphorylation and localization.

EphA2 Regulates Cell Adhesion and Proliferation. Microscopic analysis revealed that E-cadherin expression altered the adhesive profile of MDA-MB-231 cells (Fig. 8). Whereas parental and 231-neo cells were mesenchymal in appearance and readily grew atop one another, the E-cadherin-transfected cells had more prominent cell-cell adhesions and grew as single-cell monolayers. Analysis of cell-ECM[3] attachments by staining with paxillin-specific antibodies revealed numerous focal adhesions in control MDA-MB-231 cells, whereas 231-E-cad cells had fewer focal adhesions. The decrease in focal adhesions was most prominent in 231-E-cad cells within colonies (Fig. 8, *bottom right*), whereas individual cells had focal adhesions that were comparable with controls (data not shown).

EphA2 activation contributes to the decreased cell-ECM adhesion. To activate EphA2 in MDA-MB-231 cells, we aggregated EphA2 at the cell surface with specific antibodies (as described above) and found that this caused a rapid loss of focal adhesions within 5 min. This was confirmed by paxillin staining (Fig. 10) and by interference reflection microscopy (data not shown). Similar results were obtained in other neoplastic cell lines (data not shown). In contrast, treatment with either primary or secondary antibodies alone did not alter focal adhesions.

Focal adhesions are sites of intracellular signaling that promote cell growth (39, 40). Because EphA2 activation blocks focal adhesions, we questioned whether EphA2 activation would impact cell growth. To test this, we activated EphA2 with specific antibodies or B61-IgG ligand-bodies (as described above). Concomitant with receptor cross-linking, we included BrdUrd in the culture medium and measured DNA synthesis over the following 4 h. As shown in Table 1, EphA2 activation decreased the proliferation in MDA-MB-231 cells (31% reduction; $P < 0.001$), whereas control conditions (primary or secondary antibodies alone and isotype controls) did not change cell growth. The short duration of EphA2 signaling that is induced by antibody aggregation (Fig. 4*B*) likely underestimates EphA2's growth-inhibitory potential. A similar decrease in cell growth was obtained following EphA2 activation in other cell types, including MDA-MB-435 cells (22% reduction; $P < 0.0005$) and MCF-10A cells (16% reduction; $P < 0.01$). For experiments with MCF-10A, we plated cells at low cell density and scored individual cells (to preclude cell-cell contacts that might otherwise activate EphA2).

Discussion

The major findings of this study are that the localization and phosphorylation of EphA2 in mammary epithelial cells are dependent on E-cadherin-mediated adhesion and that loss of E-cadherin in metastatic tumor cells causes alterations in EphA2 localization and phosphorylation. In addition, we found that experimental induction of EphA2 phosphorylation decreases cell-ECM attachment at focal adhesions and negatively regulates the proliferation of metastatic cells.

Decreased EphA2 Phosphorylation in Metastatic Cells. We originally identified EphA2 using antibodies that recognize tyrosine-phosphorylated proteins in Ras-transformed MCF-10A-neoT cells (15). MCF-10A-neoT cells express E-cadherin (21) and, consequently, EphA2 is tyrosine-phosphorylated (data not shown). Notably, EphA2 was tyrosine-

[3] The abbreviations used are: ECM, extracellular matrix; BrdUrd, bromodeoxyuridine.

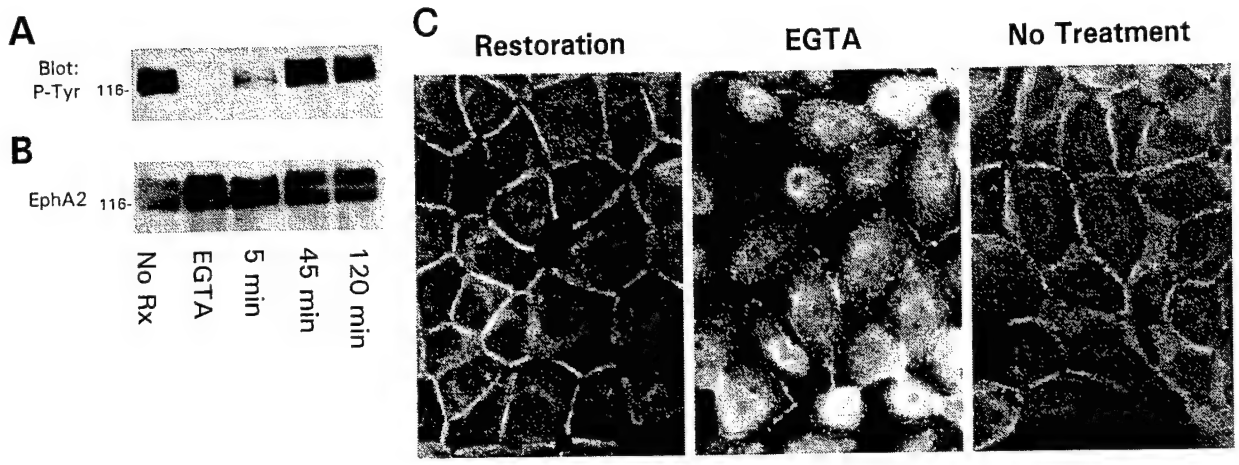

Fig. 6. EphA2 phosphorylation and localization require stable E-cadherin adhesions. Stable cell-cell contacts in monolayers of MCF-10A cells were disrupted by the addition of EGTA (4 mM, 30 min, 37°C) to the culture medium. After removal of the EGTA, normal growth medium was returned for 0–120 min. *A*, EphA2 was immunoprecipitated and Western blot analysis performed with phosphotyrosine-specific (PY20) antibodies. *B*, the blot from *A* was stripped and reprobed with EphA2 antibodies as a loading control. *C*, staining with EphA2-specific antibodies assessed changes in the subcellular distribution of EphA2 before and after restoration of cell-cell adhesions.

phosphorylated in nonneoplastic mammary epithelial cell lines but not in metastatic cell lines. In this respect, EphA2 differs from many other tyrosine kinases (*e.g.*, cErbB2, epidermal growth factor receptor, platelet-derived growth factor receptor, and Src), the phosphorylation of which increases in cancer cells (2, 41, 42). For these kinases, phosphorylation elevates tyrosine kinase activity, triggering signal transduction cascades that promote cell proliferation.

The phosphotyrosine content of EphA2 does not relate to its intrinsic enzymatic activity in mammary epithelial cells. *In vitro* assays revealed that, despite its low phosphotyrosine content, the enzymatic activity of EphA2 in metastatic cells is comparable with or increased over the activity of phosphorylated EphA2 in nonneoplastic epithelial cells. This is consistent with evidence that the phosphorylation of EphB2 also has little effect on its kinase activity (43). Our results suggest that, rather than controlling enzymatic activity, the phosphotyrosine content of EphA2 might influence the choice or availability of substrates and interacting proteins. In addition, changes in the phosphotyrosine content of EphA2 might provide signals that are independent of EphA2 enzymatic activity, which is supported by recent reports that other Eph kinases (VAB-1 and EphB2) have kinase-independent functions (44, 45). This suggests that protein interactions, localization, phosphotyrosine content, and enzymatic activity all contribute to Eph receptor function.

There are several possible explanations for the loss of EphA2 phosphorylation in metastatic cells. The primary sites of receptor autophosphorylation are not mutated because the sites that become autophosphorylated *in vitro* are the same in nontransformed and neoplastic cells.[4] Consistent with this, EphA2 tyrosine phosphorylation was restored by cross-linking EphA2 with antibodies or by transfection with E-cadherin. Another possible cause for decreased EphA2 phosphorylation could be loss of EphA2 ligands (ephrin-A class molecules). However, our ability to restore EphA2 phosphorylation in E-cadherin-transfected cells appears to exclude this possibility. A third possibility is that the phosphotyrosine content of EphA2 is repressed by an associated tyrosine-phosphatase. Consistent with this, treatment of neoplastic cells with tyrosine-phosphatase inhibitors restores normal levels of EphA2 tyrosine phosphorylation.[5] However, the identities of the phosphatases responsible for this are presently unknown.

Regulation of EphA2 Activation by E-Cadherin. We focused on the possibility that decreased stability of cell-cell contacts inhibits tyrosine phosphorylation of EphA2 in metastatic cells. Both Eph family receptor tyrosine kinases and their ephrin ligands are bound to the cell surface (1, 6, 7), so cells must be in close contact to facilitate Eph-ephrin interactions. Little is known, however, about the nature of these contacts and their precise effects on Eph-ephrin interactions.

Because many breast tumors lack E-cadherin and have unstable cell-cell junctions (18, 46), we investigated how expression of E-cadherin affects EphA2 phosphorylation in mammary epithelial cells. We found inhibition of E-cadherin function either by removal of Ca^{2+} or with function-blocking antibodies or peptides reduced EphA2 phosphorylation and caused EphA2 to redistribute into membrane ruffles. Conversely, expression of E-cadherin in MDA-MB-231 cells restored EphA2 phosphorylation and localization to sites of cell-cell contact. The simplest explanation for these results is that E-cadherin stabilizes cell-cell contacts and, thereby, facilitates interactions between EphA2 and its ligands.

At present, there is no evidence for or against a direct interaction between E-cadherin and EphA2. The two proteins are expressed in overlapping patterns. but we have not been able to coimmunoprecipitate EphA2 and E-cadherin.[5] EphA2

[4] M. S. Kinch, unpublished results.

[5] N. D. Zantek, unpublished results.

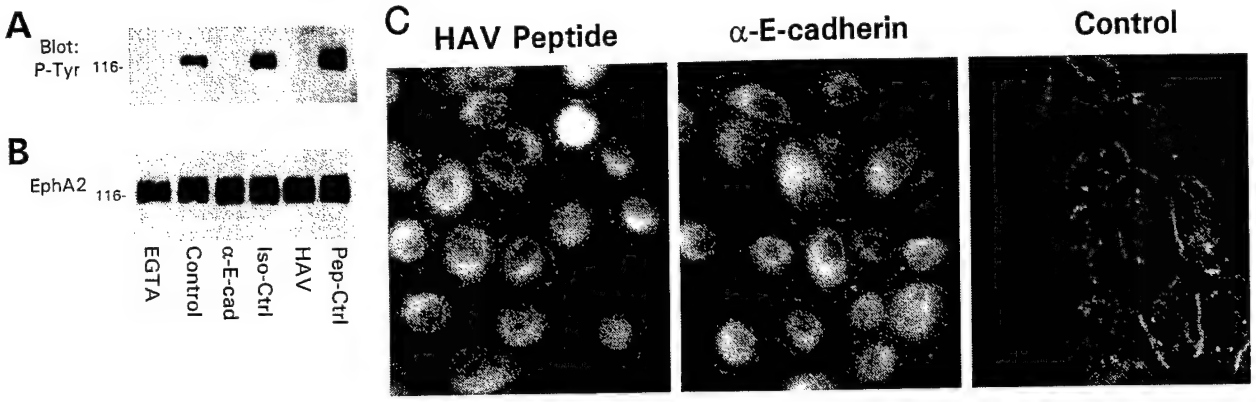

Fig. 7. Inhibition of E-cadherin-mediated adhesion. Following treatment of MCF-10A cell monolayers with EGTA, normal medium conditions were restored in the absence (*Control*) or presence of function-blocking E-cadherin antibodies (*α-E-cad*) or peptides (*HAV*). Isotype control antibodies (*Iso-Ctrl*) and scrambled peptides (*Pep-Ctrl*) were included as matched negative controls. *A*, immunoprecipitated EphA2 was subjected to Western blot analysis with phosphotyrosine (PY20) antibodies. *B*, the same blot as in *A* was stripped and reprobed with EphA2 antibodies as a loading control. *C*, EphA2 localization was determined after calcium restoration in the absence (*Control*) or presence of E-cadherin inhibitors.

also does not cocluster with E-cadherin at the cell surface in response to antibody-mediated aggregation of either molecule,[6] which is consistent with our biochemical evidence. We cannot exclude that experimental conditions used for protein extraction dissociate such interactions or that a small fraction of activated EphA2 coclusters with E-cadherin. Direct interaction between the two molecules may not be necessary if E-cadherin primarily serves to stabilize cell-cell contacts and thereby promote interactions between EphA2 and its ligands. Other aspects of E-cadherin function, such as signaling (28), cytoskeletal association (47), and junction formation (16) might also target EphA2 to sites of cell-cell contact.

EphA2 Regulates Cell-ECM Adhesion and Growth. An immediate consequence of EphA2 activation is decreased cell-ECM contact at focal adhesions. Focal adhesions are sites of membrane-cytoskeletal interaction that provide anchorage for cell migration and invasion (48). Focal adhesions also play critical roles in signal transduction, where they organize intracellular signals that control cell growth and survival (39, 40). We propose that E-cadherin-mediated stabilization of ligand binding induces EphA2 to block focal adhesions. Consistent with this, it is understood that epithelial cells balance their cell-cell and cell-ECM adhesions and that this is linked with the proper functioning of E-cadherin (49, 50). Individual epithelial cells have more focal adhesions than cells within colonies, whereas cells with decreased E-cadherin function have increased cell-matrix adhesion, regardless of cell density (21). Although the molecular mechanisms responsible for this are unknown, many proteins that interact with Eph kinases regulate cell adhesion or cytoskeletal organization, including the p85 subunit of phosphatidylinositol 3′-kinase, Src, Fyn, and Ras-GAP (35, 51–53).

Focal adhesions initiate signals that promote cell growth, and it follows that loss of these structures may contribute to decreased cell growth following EphA2 activation. By inference, loss of EphA2 activation might contribute to deregulated growth of neoplastic cells by increasing signals from focal adhesions. This would be consistent with evidence that neoplastic cells have increased signaling by focal adhesion proteins (*e.g.*, FAK; Ref. 54). Although EphA2 activation decreases cell growth, the expression pattern of EphA2 does not fit the classic pattern of a tumor suppressor. Most tumor suppressors are inactivated either because of decreased expression or loss of enzymatic activity. In contrast, neoplastic cells express high levels of EphA2, which, although nonphosphorylated, retains comparable levels of enzymatic activity. An alternative explanation is that EphA2 positively regulates cell growth but that this signaling is reduced in nontransformed epithelia. Support for this includes evidence that EphA2 is overexpressed in neoplastic cells and is supported by the fact that other Eph kinases (*e.g.*, EphA1) are oncogenic (55). In this scenario, EphA2 "activation" by E-cadherin or receptor aggregation might decrease EphA2 function, perhaps by reducing EphA2 expression levels. It is intriguing that the lowest levels of EphA2 are found in cells where it is phosphorylated and that ligand-mediated aggregation decreases EphA2 expression levels. A third possibility is that EphA2 functions very differently in normal and neoplastic epithelia. The phosphotyrosine content and subcellular localization of EphA2 differ in normal and neoplastic cells, and either property could alter substrate specificity or availability. Indeed, tyrosine-phosphorylated EphA2 (but not unphosphorylated EphA2) interacts with the phosphatidylinositol 3′-kinase and the SLAP adapter protein (56). SLAP was recently shown to negatively regulate cell growth (57), which is supportive of our evidence that EphA2 also regulates cell proliferation. Future studies will be necessary to define EphA2's role as a positive and/or negative regulator of cell growth and to determine whether these properties differ between normal and neoplastic epithelia.

Conclusions. Loss of E-cadherin in carcinomas promotes invasion (18, 58), cell motility (27), and cell proliferation (26). In this study, we have identified the receptor tyrosine kinase EphA2 as one protein that is phosphorylated after cell-cell contact and demonstrated that both the phospho-

[6] M. Fedor-Chaiken and M. S. Kinch, unpublished results.

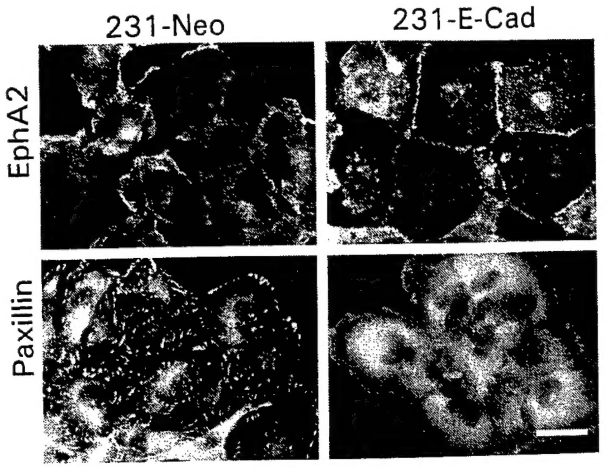

Fig. 8. E-Cadherin expression directs EphA2 into cell-cell contacts. The subcellular distribution of EphA2 and paxillin was assessed by immunofluorescence microscopy in control (*231-Neo*) and E-cadherin transfected (*231-E-Cad*) MDA-MB-231 cells. Note that E-cadherin promotes a redistribution of EphA2 into cell-cell contacts and decreases focal adhesions. *Scale bar*, 25 μm.

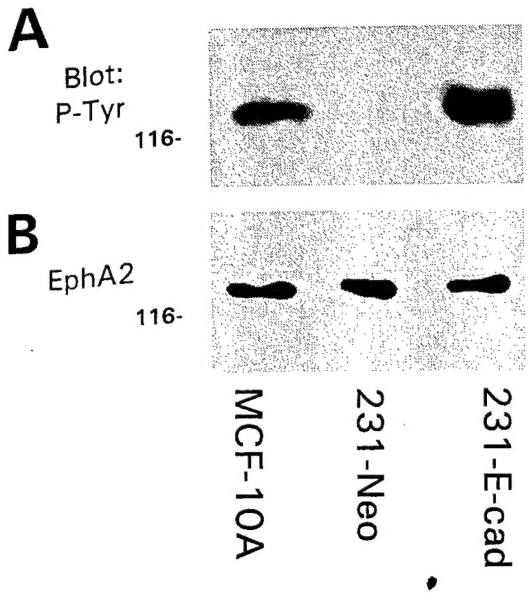

Fig. 9. E-cadherin expression restores normal EphA2 function. *A*, the phosphotyrosine content of immunoprecipitated EphA2 was measured by Western blot analysis following transfection of MDA-MB-231 cells with E-cadherin (*231-E-cad*) or a matched vector control (*231-Neo*). MCF-10A was included as a positive control for EphA2 tyrosine phosphorylation. *B*, The blot from *A* was stripped and reprobed with EphA2-specific antibodies as a loading control.

rylation and localization of EphA2 are sensitive to changes in E-cadherin function and expression. We also find that EphA2 activation negatively regulates cell-ECM adhesion and cell growth. These findings raise the possibility that important effects of E-cadherin on tumor cell behavior may occur via effects on EphA2.

Materials and Methods

Cell Lines and Antibodies. Human breast carcinoma cells and nontransformed human mammary epithelial cell lines were cultured as described previously (29, 46). We purchased antibodies specific for E-cadherin (polyclonal antibodies, Transduction Laboratories, Lexington, KY; and DECMA-1, Sigma Chemical Co., St. Louis, MO), phosphotyrosine (PY20, ICN, Costa Mesa, CA; 4G10, Upstate Biotechnology Inc., Lake Placid, NY; and polyclonal antibodies, Transduction Laboratories), and fluorescein-conjugated BrdUrd (Harlan Sera-Lab Ltd., Loughborough, United Kingdom). Monoclonal antibodies specific for EphA2 (clones D7 and B2D6) were produced in the laboratory as described (15) or purchased from Upstate Biotechnology Inc. Rabbit polyclonal antibodies for EphA2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). EK166B monoclonal EphA2 antibodies were generously provided by R. Lindberg (Amgen, Thousand Oaks, CA). Paxillin-specific antibodies were obtained from K. Burridge (University of North Carolina, Chapel Hill, NC). To visualize f-actin, we used fluorescein-conjugated phalloidin, purchased from Molecular Probes (Eugene, OR).

Western Blot Analysis. Unless noted otherwise, all experiments used confluent cell monolayers that were extracted in a buffer containing 1% Triton X-100 or in RIPA buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS for 6 min on ice, as described previously (21). After protein concentrations were measured by Coomassie Blue staining (Pierce, Rockford, IL) or Bio-Rad D_C Protein Assay (Hercules, CA), equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose (Protran, Schleicher & Schuell, Keene, NH), and Western blot analysis was performed as described previously (21). Antibody binding was detected by enhanced chemiluminescence as recommended by the manufacturer (Pierce). To reprobe, we stripped blots as described previously (21).

Immunofluorescence and Confocal Microscopy. Immunostaining was performed as described previously (21). In brief, cells were grown on glass coverslips to visualize individual cells. Cells were observed at both high cell density (~70% confluence) and low cell density (~20% confluence) by seeding 1×10^6 cells onto either a 3.5- or 10-cm tissue culture plate overnight at 37°C. At high cell density, extensive overlapping of neoplastic cells precludes accurate subcellular visualization. The samples were fixed in 3.7% formaldehyde solution, extracted in 0.5% Triton X-100, and stained. Immunostaining was visualized using rhodamine-conjugated donkey antimouse antibodies (Chemicon, Temecula, CA) and FITC-conjugated donkey antirabbit (Chemicon) and epifluorescence microscopy (model BX60, ×600, Olympus Lake Success, NY) and recorded onto T-Max 400 film (Eastman-Kodak, Rochester, NY). For confocal microscopy, samples were viewed on a Nikon Diaphot 300 outfitted with a Bio-Rad MRC 1024 UV/Vis System and Coherent Innova Enterprise model 622 60-mW output water-cooled lasers.

Immunoprecipitation. Immunoprecipitation experiments were performed as described (21) for 1.5 h at 4°C with the appropriate EphA2-specific monoclonal antibodies (D7 or B2D6) and rabbit antimouse (Chemicon) conjugated protein A-Sepharose (Sigma). Immunoprecipitates were washed three times in lysis buffer, resuspended in SDS sample buffer (Tris buffer containing 5% SDS, 3.8% DTT, 25% glycerol, and 0.1% bromphenol blue), and resolved by 10% SDS-PAGE.

***In Vitro* Kinase Assays.** For *in vitro* autophosphorylation assays, immunoprecipitated EphA2 was washed in lysis buffer and incubated in 10 mM PIPES, 3 mM $MnCl_2$, 5 mM PNPP (Sigma 104 phosphatase substrate; Sigma), 1 mM $NaVO_4$, 1 μM ATP, and 10 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, MA) at 25°C for the times shown. The reactions were terminated by the addition of 5× Laemmli sample buffer at multiple time points before saturation. After resolving samples by 10% SDS-PAGE, the gel was transferred to nitrocellulose (Schleicher & Schuell) or Immobilon P (Pierce), and incorporated material was detected by autoradiography. To hydrolyze phosphoserine/threonine, we treated the membranes with 1 N KOH at 65°C for 1 h and reassessed them by autoradiography. After several half-lives, Western blot analysis was performed to determine EphA2 loading.

Cross-Linking of EphA2 Receptors. For antibody cross-linking experiments, cells grown as a monolayer were incubated at 4°C for 20 min with 4 μg/ml EphA2 antibody (either clone EK166B or B2D6) or purified fusion protein of ephrin-A1 fused to IgG (B61-IgG; Ref. 10). Primary antibody alone, rabbit antimouse IgG alone and control fusion proteins were used as controls. The samples were washed with medium, incubated with 20 μg/ml rabbit antimouse IgG in conditioned medium at 4°C

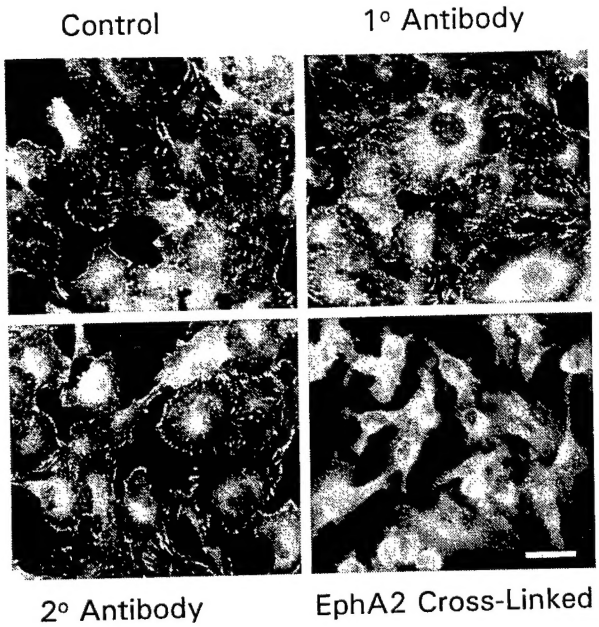

Fig. 10. EphA2 activation decreases cell-ECM adhesion. The presence of focal adhesions was assessed by immunostaining for paxillin in MDA-MB-231 cells before and after activation of EphA2 by antibody-mediated aggregation. Note that incubation of cells with either primary (*1°*) or secondary (*2°*) antibodies alone did not alter the presence of focal adhesions, whereas EphA2 aggregation dissipated focal adhesions. *Scale bar*, 25 μm.

Table 1 EphA2 Activation Inhibits Cell Proliferation[a]

Cell line	Treatment	% BrdUrd uptake (mean ± SE)	Statistical analysis[b] (*P*)
MDA-MB-231	Untreated	43.8 ± 2.0	
	Primary Ab[c] alone	44.1 ± 2.2	>0.43
	Secondary Ab alone	39.7 ± 2.3	>0.21
	Primary + secondary	30.4 ± 1.7[d]	<0.0001[d]
	Control-IgG + secondary	43.0 ± 2.1	>0.44
	B61-IgG + secondary	29.1 ± 3.1[d]	<0.01[d,e]
MDA-MB-435	Untreated	52.8 ± 5.1	
	Primary Ab alone	52.6 ± 3.4	>0.25
	Secondary Ab alone	52.8 ± 6.3	>0.39
	Primary + secondary	39.6 ± 3.0[d]	<0.00005[d]
MCF-10A (low density)	Untreated	53.6 ± 1.8	
	Primary Ab alone	53.9 ± 0.8	>0.43
	Secondary Ab alone	55.1 ± 0.5	>0.22
	Primary + secondary	45.0 ± 1.4[d]	<0.01[d]

[a] BrdUrd uptake into newly synthesized DNA was measured for 4 h after cross-linking of EphA2 at the cell surface with specific antibodies. The data represent at least three independent, double-blinded experiments. Cell growth was determined in at least 100 cells from each experimental and control, and the results shown are compared with DNA synthesis with untreated (untreated) samples. None of the differences between or among individual negative controls (untreated, primary antibody alone, or secondary antibody alone) were significant ($P > 0.05$).
[b] Statistical analyses compared the experimental to untreated for each sample.
[c] Ab, antibody.
[d] ●●●.
[e] For the fusion proteins, there was also a significant difference ($P < 0.02$) between the control and B61 fusion proteins.

for 10 min, and warmed to 37°C for 10 min before extraction and immunoprecipitation. To determine the optimal time for activation, we incubated the plates in the presence of cross-linking antibody at 37°C for 0–120 min.

EGTA and Antibody Treatments. "Calcium switch" experiments were performed as described previously (28). Monolayers of MCF-10A cells were grown to ~80% confluence. EGTA was added to growth medium to a final concentration of 4 mM, and the cells were incubated at 37°C for 30 min. The medium was removed, and calcium concentrations restored with normal growth medium. To block E-cadherin function, we supplemented the medium with E-cadherin antibodies (1:100 dilution; DECMA-1; Sigma) or 10 μg/ml peptide corresponding to the E-cadherin HAV sequence (YTLFS*HAV*SSNGN). Controls include isotype control antibodies (rat anti-HA antibody; Boehringer Mannheim, Indianapolis, IN) and matched, scrambled peptides (SGATNSLHNFSVY). The Purdue Laboratory for Macromolecular Structure synthesized peptides. Cells were then incubated for the indicated times at 37°C and extracted for Western blot analysis and immunoprecipitation. Cell monolayers grown on glass coverslips were treated in the same manner and immunostained for EphA2.

E-Cadherin Expression and Function. MDA-MB-231 cells were co-transfected with pBATEM2, a mouse E-cadherin expression vector (59) and pSV2neo (60) using FuGENE 6 Transfection Reagent (Boehringer Mannheim), following the manufacturer's instructions. Transfected cells were selected in growth media supplemented with 400 μg/ml G418. Immunostaining and Western blot analysis with specific antibodies confirmed E-cadherin expression.

Proliferation Assay. Cells were plated onto glass coverslips and cultured overnight in growth medium. EphA2 antibodies (EK166B or B2D6, extracellular or D7, intracellular) or ligand fusion protein (B61-IgG) were added to the media at 1 μg/ml and incubated at 4°C for 20 min, washed with medium, and incubated with 20 μg/ml rabbit antimouse plus 3 μg/ml BrdUrd at 37°C for 4 h. Cells were fixed in cold methanol for 8 min, extracted with 2 N HCl at 37°C for 30 min and stained with a BrdUrd antibody to indicate proliferating cells and Hoechst dye to label the nuclei of all cells on the coverslip. A minimum of six random fields were selected in a double-blind study, and at least 150 cells were assessed in each sample. Each experiment was repeated at least three times.

Statistical Methods. All statistical analyses were performed using the SAS System for Windows, Version 6.12. An ANOVA model was used to compare the percentage of cells that grew in each field, within each specimen, in the control group to the percentage of cells that grew in each field, within each specimen, in the experimental group. Group (control *versus* experimental) was treated as a fixed effect and specimen within each group was treated as a random effect. A normal probability plot of the residuals was used to assess the homogeneity of the variances of the mean percentage cell growth for the control and experimental groups. $P < 0.05$ was considered statistically significant.

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